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Benson H. Morrill
Utah State University

HISTORICAL BIOGEOGRAPHY OF LOWLAND SPECIES OF TOADS (*BUFO*) ACROSS THE TRANS-MEXICAN NEOVOLCANIC BELT AND THE ISTHMUS OF TEHUANTEPEC

by

Benson H. Morrill

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Abstract: The eastern extension of the massive Trans-Mexican Neovolcanic Belt (TMNB) contacts the Atlantic Coast in central Veracruz, Mexico. Although it is not a massive structure at this eastern terminus, the TMNB has nonetheless effected vicariance and subsequent speciation in several species groups, including bufonid toads and freshwater fishes. In this study, we examined mtDNA sequences (cyt b, 16S) from populations of two different species groups of toads (*B. marinus*, and the *B. nebulifer–B. valliceps* species pair) from a lowland transect across the eastern end of the TMNB. We also included samples from outside this region (e.g., Costa Rica, Honduras, northern Mexico) for both species groups. Our results further define the parapatric contact zone between the Pliocene-vicariant, sister-species *B. nebulifer* and *B. valliceps*. In contrast, we found no significant phylogenetic structure among populations of *B. marinus* across either side of the TMNB, suggesting a more recent Pleistocene dispersal in this group. In addition, we found phylogenetic structure associated with the Isthmus of Tehuantepec in both species groups. The Isthmus of Tehuantepec unites the North American continent with nuclear Central America, and the presence of a Pliocene seaway across this region has been controversial. Our data support clades on either side of the isthmus within two relatively unrelated species (*B. valliceps* and *B. marinus*). Both of these species contain clades that are consistent with a late Pliocene divergence, which supports the hypothesis of a marine barrier across the Isthmus of Tehuantepec at this time. The divergence between *B. valliceps* and *B. nebulifer* across the TMNB is further supported, and appears

to predate the isthmian break in this complex, whereas the *B. marinus* group shows a dispersal across the TMNB subsequent to the presence of a late-Pliocene seaway over the Isthmus of Tehuantepec.

The Trans-Mexico Neovolcanic Belt (TMNB) is one of the predominant geographical features of Mexico, and its geological development has been posited as a primary contributor to the biogeographic histories of many upland taxa in central Mexico (e.g., Campbell and Frost, 1993; Darda, 1994; Parkinson et al., 2000; Sullivan et al., 2000; Castoe et al., 2003). However, a recent series of papers (Mulcahy and Mendelson, 2000; Hulsey et al., 2004; Zaldívar-Riverón et al, 2004) have independently demonstrated the considerable influence of this transverse massif on the biogeography and evolution of the lowland fauna along the central Atlantic Versant of Mexico. These papers generally support an earlier hypothesis implied by Pérez-Higareda and Navarro (1980): based on their observation of consistent disjunctions in the distribution of subspecies (reptiles and mammals) along the Atlantic Coast, they suggested that the TMNB may form a geographical barrier to lowland species in the region.

The concept of a massive volcanic chain such as the TMNB acting as a vicariant feature to lowland populations is easily tractable, but the reality is that the TMNB withers to a tiny string of lava-rock strewn hills at its eastern terminus. It makes final contact with the current coastline, as two small fingers of raised lava-rock, just south of the town of Palma Sola, Veracruz, Mexico. Unlike the imposing backbone of the TMNB, which boasts the highest peaks of Mexico such as Pico de Orizaba and Cofre de Perote, the eastern fingers of the TMNB are barely noticeable to the casual traveler in the region. Mulcahy and Mendelson (2000) recounted the history of seawater inundations in this

region that included Miocene–Pliocene-era maxima that effectively covered the entire coastal plain of central Mexico and Pleistocene-era minima that exposed large sections of the continental shelf to greatly increase the areal extent of the coastal plain. By comparing phylogenetic signal from mtDNA sequences of lowland toads in this region, Mulcahy and Mendelson (2000) demonstrated that the prevailing concept of a wide-ranging single species (*Bufo valliceps* Wiegmann) was actually a species pair showing an apparent parapatric distribution in the region: *B. valliceps* ranging from central Veracruz, Mexico, southward to Costa Rica; and *B. nebulifer* Girard, ranging from central Veracruz northward to the southern United States. Mulcahy and Mendelson (2000) proposed and tested two historical hypotheses related to the timing of this speciation event: 1) Miocene-Pliocene vicariance associated with the orogeny of the TMNB; and 2) Pleistocene vicariance and dispersal associated with raised sea levels that obliterated the coastal plain in this region; their results supported the Miocene–Pliocene vicariance hypothesis. However, their sampling was not sufficient enough to allow them to demonstrate strict parapatry on the narrow coastal plains on either side of the TMNB. In this paper we use recently collected samples from a geographic transect in the central part of coastal Veracruz, Mexico, to test the hypothesis of parapatry of *B. nebulifer* and *B. valliceps* across the eastern terminus of the TMNB (Mulcahy and Mendelson, 2000). We also propose and test a related hypothesis that the sympatric toad *B. marinus* shows a more recent dispersal across the TMNB, consistent with the Pleistocene vicariance and dispersal hypothesis of Mulcahy and Mendelson (2000). We test this hypothesis based on the fact that *B. marinus* is from South American stock, and most likely entered lower Central America during the Pliocene. A broad scale phylogeographic study of *B.*

marinus (Slade and Moritz, 1998) indicated that this complex showed dramatic historical effects of the orogeny of the Andes in South America, and some level of genetic divergence between samples from Costa Rica and Mexico. These results suggest that, in our finer scale study, the biogeographic history of northern *B. marinus* may show an influence of the TMNB similar to that already documented in other toads in the region (i.e., *B. valliceps* and *B. nebulifer*).

The three species of toads in our study are ecologically similar in their general reproductive biology and overall natural history. All three species are invasive, "weedy" species that are typically more abundant in secondary, degraded habitats than in undisturbed primary forests (Mendelson, 1994). These attributes would suggest that they are suitably comparable to one another, in order to test hypotheses of historical biogeography, and also that their invasive, dispersalist tendencies would make them a conservative test of any potential vicariant effect of the TMNB on lowland species.

In this project we use our study system to address three principal questions regarding the historical biogeography of the Atlantic versant lowlands of Mexico: 1) the parapatry of *B. valliceps* and *B. nebulifer* at the the eastern terminus of the TMNB; 2) evidence of phylogenetic breaks within *B. valliceps* and/or *B. marinus* that are consistent with hypothesized seaways across the Isthmus of Tehuantepec; 3) evidence of Pleistocene dispersal of *B. marinus* across the TMNB.

Materials and Methods

Sequences were examined from 80 individuals which included 75 ingroup specimens (i.e., *B. marinus B. nebulifer*, and *B. valliceps*). Outgroup taxa for *B. marinus* included *B. crucifer*, *B. paracnemis*, and a South American *B. marinus*. Outgroup taxa

for *B. nebulifer* and *B. valliceps* included *B. campbelli*, and *B. mazatlanensis*. Outgroup taxa were chosen following phylogenetic hypotheses of Graybeal (1997) and Mulcahy and Mendelson (2000, unpublished data). Because some ingroup specimens were identical for the regions of the genes examined, one sequence for each haplotype from every locality sampled was used in the phylogenetic analysis. Thus, our final data set contains a haplotype from every locality sampled, and five outgroup samples. Voucher specimens for this study are deposited at the following institutions: The University of Texas at Arlington (UTA); Museo de Zoologia, Facultad de Ciencias, Universidad Autonoma de Mexico (MZFC); Museum of Vertebrate Zoology, University of California, Berkeley (MVZ); and the United States National Museum (USNM).

Laboratory Protocols

In the family Bufonidae, the ribosomal gene 16S and protein-coding gene cyt b have been demonstrated to be accurate markers for resolving recent divergences (Graybeal, 1997, 1993). For all specimens, tissue was taken from liver, muscle, skin, or toe clips and stored either at –80C or in 95% ethanol. Extraction of DNA followed standard phenol/chloroform extraction methods (Maniatis et al., 1982). Primers used for PCR amplification and sequence reactions (for cyt b, MVZ43: GAGTCTGCCT[A/T]AT[T/C] GC[C/T]CA[A/G]AT, 3' and MVZ28: CGAGGC[G/C]CC[T/C]GCAAT[A/G]ATAA, 3'; for 16S, 16Sar: CGCCTGTTTATCAAAAACAT, 3' and 16Sbr: CCGGTCTGAACTCAGATCACGT 3') followed recipes for cyt b and 16S from Graybeal (1993 and 1997 respectively) and were purchased from Integrated DNA Technologies Inc. (IDT). Amplifications for PCR

were done in 50-ul reactions using 2% Taq DNA polymerase, 10% buffer, and 10% MgCl, 20% dNTP's (100uM), 5% of each primer (10uM), and 2-10% of DNA template depending on concentration as determined by running on agarose gel with a concentration standard and ladder. Polymerase chain reactions were performed in a Minicycler (MJ Research) thremocycler under the following profile: hot start at 94C for 5 min, 30 cycles of denature at 95C for 1.5 min, annealing at 55C for 1.5 min, elongation at 72C for 3 min, and a final elongation at 72C for 5 min.

Each primer for cyt b and 16S amplifies approximately 400- and 560-bp fragments, respectively, and each gene has a primer from the 5' and the complimentary 3' direction. Therefore, no internal primers were needed and all nucleotides were confirmed by sequences from both directions. Actual sequence reactions and sequencing were done using an ABI 377 automated sequencer. Sequence comparisons and alignment were done using Sequencher 3.1 and macClade 3.05 (Maddison and Maddison, 1998).

Phylogenetic Analysis

Pylogenetic analyses, percent sequence-divergence, and base frequencies were calculated with PAUP* 4.0b2a (Swoffored, 1999). Maximium-parsimony (MP) analysis was conducted on both genes combined for each sample. MP analysis was done using ACCTRAN character-state optimization and heuristic search option. Branch-swapping algorithms for all analyses used Tree-bisection-reconnection and random-addition sequence with 100 replicates. Non-parametric bootstrap analysis was used to assess branch support in the MP analysis (100 replicates).

Nucleotide-sequence data, as opposed to amino acid translations, were used for the character matrix based on the low number of amino acid differences and the more numerous synonymous substitutions (e.g., third codon positions; see Graybeal, 1993, 1997). Character states were treated as unordered, and gaps were treated as missing data.

Results

Genetic Variation

For all the *B. valliceps* and *B. nebulifer* specimens used in the analyses, fragments of the two mtDNA genes were 562 and 421 bp for 16S and cyt b, respectively. The gene regions used in all analyses correspond to the gene regions chosen by Mulcahy and Mendelson (2000). This combination resulted in a total of 983 bp in the *B. valliceps* and *B. nebulifer* data set. In this study, we used fragments of 16S and cyt b genes for 19 individuals of *B. valliceps* and *B. nebulifer* that were not used by Mulcahy and Mendelson (2000). These 19 additional samples added 15 new unique haplotypes, and represented 4 new localities. We combined these data with those from Mulcahy and Mendelson (2000) to include one unique sequence from every locality sampled, producing 50 ingroup OTU's (35 unique haplotypes from 27 different localities), and 3 outgroup taxa.

For the specimens of *B. marinus*, fragments of the two mtDNA genes were 556 and 417 bp for 16S and cyt b, respectively. The gene regions used in all analyses correspond to the gene regions chosen in Mulcahy and Mendelson (2000). This combination resulted in a total of 973 bp in the *B. marinus* data set. In this study, we sequenced fragments of 16S and cyt b genes for 23 samples of *B. marinus*. We combined

the data to include one unique sequence from every locality sampled, producing 29 ingroup OTU's (19 unique haplotypes from 14 different localities), and 3 outgroup taxa.

Phylogenetic Analyses

In the MP analysis of *B. valliceps* and *B. nebulifer*, 85 of the 983 total bp were variable and 65 characters were parsimony informative; the analysis produced 2 trees. In the MP analysis of *B. marinus*, 72 of the 830 total bp were variable and 71 characters were parsimony informative; the analysis of *B. marinus* produced 60 trees. The MP analysis of *B. valliceps* and *B. nebulifer* (Fig. 1) revealed all samples of each species to represent monophyletic groups; bootstrap support for these clades was 95% for *B. valliceps* and 99% for *B. nebulifer*. Two well-supported subclades within *B. valliceps* were also recovered; these represent geographic samples from either side of the Isthmus of Tehuantepec. There seems to also be evidence that similar subclades from either side of the Isthmus of Tehuantepec exist in *B. marinus* as well, but further sampling must be done in order to see if such phylogenetic structure does exist.

Discussion

The phylogenetic analysis of the *B. valliceps* and *B. nebulifer* samples (Fig. 1) support the findings of Mulchahy and Mendelson (2000), indicating that *B. valliceps* and *B. nebulifer* are two separate monophyletic groups, representing distinct northern and southern clades (99 and 95 bootstrap values for north and south, respectively). The northern clade shows a stepwise-ladder pattern diverging from south to north, as was also

found by Mulcahy and Mendelson (2000). Within the southern clade there seems to be two subclades separated by the Isthmus of Tehuantepec.

The analyses for the *B. marinus* samples provide evidence that the uprising of the TMNB did not cause a vicariant event in *B. marinus*, as our data suggest was the case in the species pair of *B. valliceps* and *B. nebulifer*. Also, both the *B. valliceps* and *B. marinus* data show phylogenetic structure associated with the Isthmus of Tehuantepec. These data suggest that further sampling of both *B. valliceps* and *B. marinus* should be done across this isthmus in order to better test the hypothesis of historical separation on either side of the region.

Our data indicate that although these three species of toads have very similar natural histories, the formation of the TMNB affected their ability to disperse differently. Our data suggest that the ancestor of the *B. valliceps–B. nebulifer* species pair was in place along the Pacific lowlands of central Veracruz, and experienced an event classic vicariant speciation associated with the Miocene–Pliocene orogeny of the TMNB. The current parapatric distribution of these two species—and the absence of evidence of hybridization between them—indicates that the TMNB remains a barrier to these toads. In the case of *B. marinus*, our data suggest that it either had not yet dispersed to this region when the TMNB was forming, or that the orogeny had no effect on local populations (i.e., subsequent dispersal across the barrier precluded speciation). We have no evidence that the current remnant of the TMNB in the study region forms a barrier to *B. marinus*. In any case, these data are testament to the remarkable and infamous dispersalist abilities of *B. marinus*, even more so than its lowland congeners *B. valliceps* and *B. nebulifer*. All of our analyses suggest the novel hypothesis of a historical vicariant

influence in the region of the Isthmus of Tehuantepec. The patterns we observed are consistent with the existence of an ancient seaway across the current isthmus. These hypotheses warrant substantial further inquiry.

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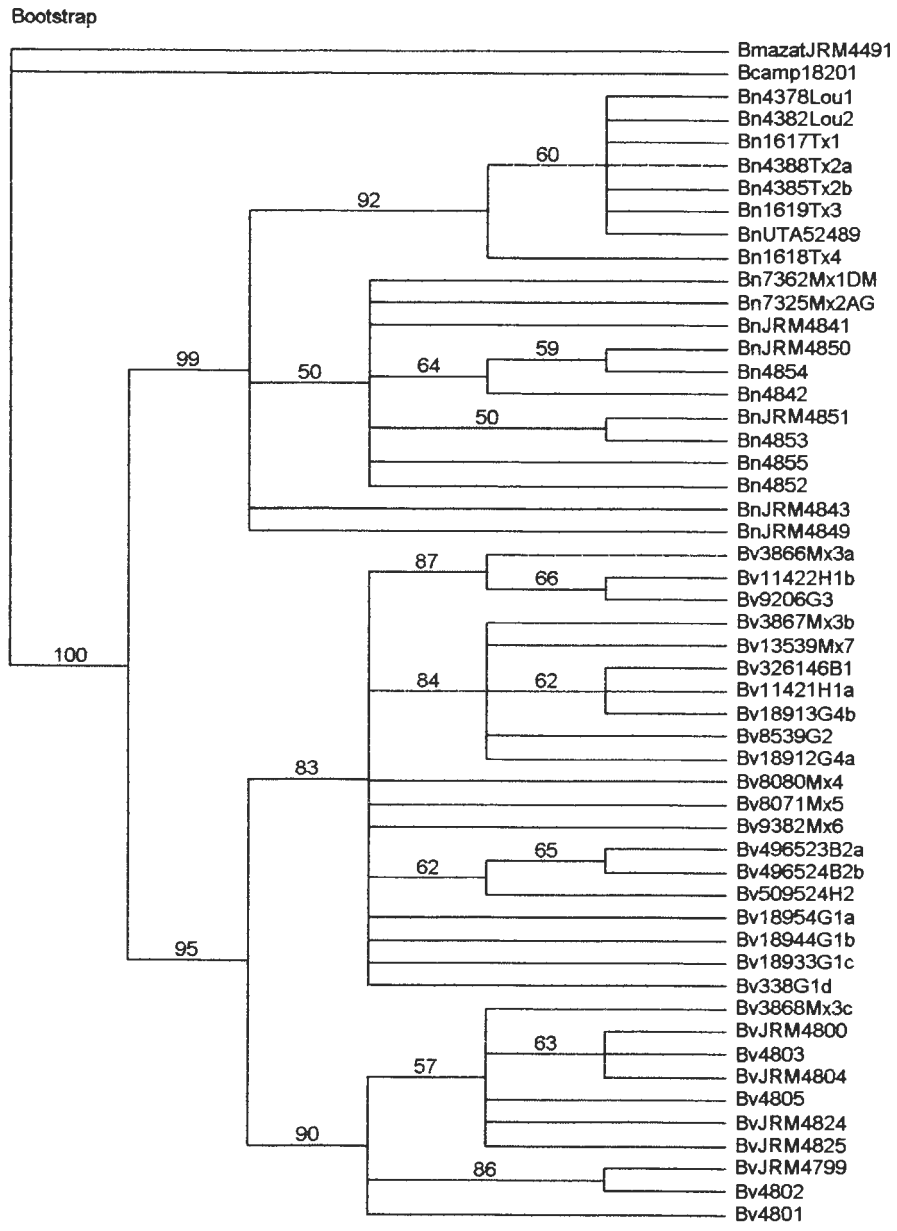

Figure 1. Results of the Maximum Parsimony analysis of samples of *Bufo valliceps* and *B. nebulifer* from a transect across the TMNB. Bootstrap values, based on 100 replicates, are shown for branches with greater that 50% support.

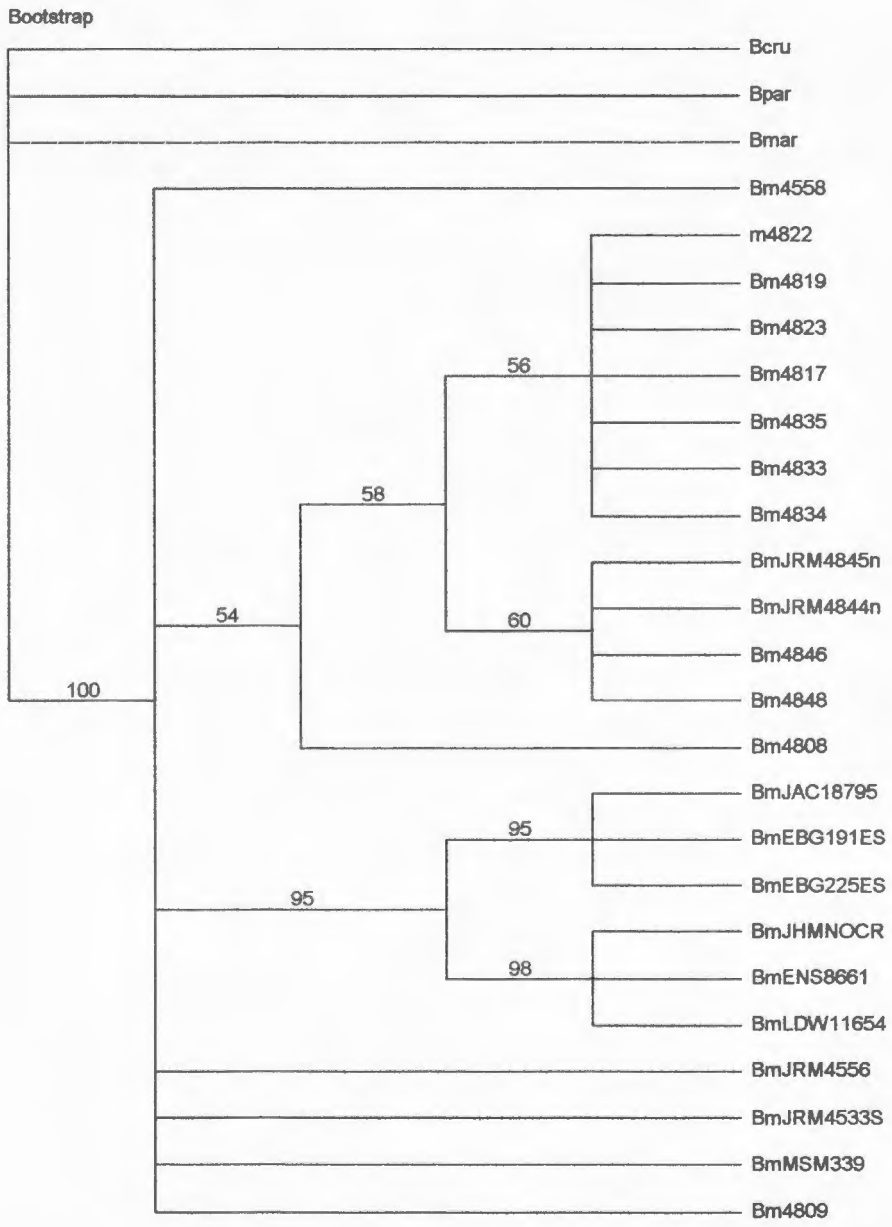

Figure 2. Results of the Maximum Parsimony analysis of samples of *Bufo marinus* from a transect across the TMNB, and also including samples from a broader region of Central America. Bootstrap values, based on 100 replicates, are shown for branches with greater that 50% support.